Biomaterials 32 (2011) 7961–7970

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Surface camouflage of pancreatic islets using 6-arm-PEG-catechol in combined therapy with tacrolimus and anti-CD154 monoclonal antibody for xenotransplantation

Jee-Heon Jeong [a], Sung Woo Hong [b], Seonki Hong [c], Simmyung Yook [a], Yoonsuk Jung [a], Jun-Beom Park [b], Cao Duy Khue [a], Bok-Hyeon Im [a], Jinwon Seo [a], Haeshin Lee [c,d], Cheol-Hee Ahn [e], Dong Yun Lee [f,1,**], Youngro Byun [a,b,1,*]

[a] Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, South Korea
[b] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, South Korea
[c] Department of Chemistry, College of Natural Science, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea
[d] Graduate School Nanoscience and Technology, College of Natural Science, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea
[e] Research Institute of Advanced Materials, Department of Materials Science and Engineering, Seoul National University, Seoul 151-742, South Korea
[f] Department of Bioengineering, College of Engineering, Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, South Korea

ARTICLE INFO

Article history:
Received 2 May 2011
Accepted 28 June 2011
Available online 9 August 2011

Keywords:
Pancreatic islets
Surface camouflage
6-arm-PEG-catechol
Anti-CD154 mAb
FK506

ABSTRACT

This study proposes a new combination method of using 6-arm-PEG-catechol to enhance the PEG effect on one hand and another combination of using low doses of Tacrolimus (FK506) and anti-CD154 mAb (MR1) with PEGylation for effective immunoprotection on the other in a xenogenic islet transplantation model. The surface coverage of PEG, viability and functionality of islets were evaluated *in vitro*, and the effect of surface camouflage on immunoprotection for transplanted islets was evaluated. In addition, the synergistic effects of surface camouflaged islets with low doses of immunosuppressant drugs, such as FK506 and MR1, were evaluated in the xenotransplantation model. The median survival time (MST) of 6-arm-PEG-catechol grafted islets (12.0 ± 1.1 days) was not significantly increased, compared to that of unmodified islets (10.5 ± 1.3 days). However, when 0.2 mg/kg of FK506 was daily administered, the MST of 6-arm-PEG-catechol grafted islet (21.0 ± 1.9 days) was increased twice, compared to that of unmodified islets treated with 0.2 mg/kg of FK506 (10.0 ± 0.9 days). Interestingly, when the recipients of 6-arm-PEG-catechol grafted islets were treated with 0.2 mg/kg of FK506 and 0.1 mg/mouse of MR1, normoglycemia was maintained up to 50 days of transplantation without any fluctuation of glucose level. Therefore, a newly developed protocol using 6-arm-PEG-catechol with FK506 and MR1 would certainly be an effective combination therapy for the treatment of type 1 diabetes.

1. Introduction

Pancreatic islet transplantation is a promising method to treat type 1 diabetes, albeit transplanted islets are susceptible to rejection by the immune reaction. To protect the transplanted islets against immune reactions, several kinds of immunosuppressant drugs such as tacrolimus and sirolimus should be administered. In this regard, the 'Edmonton protocol' has been accepted as one of the best protocols outlined for clinical islet transplantation [1,2]. However, a long-term immunosuppressive therapy is accompanied by several adverse effects such as nephrotoxicity, neurotoxicity, infectious disease, hypertension, etc. [3–5]. In addition, Shapiro et al. reported that only below 10% of patients under the Edmonton protocol achieved insulin independence after 5 years of transplantation [1]. Thus, a new immunosuppressive remedy has been in need to enhance the immunoprotection efficacy with reducing adverse effects of immunosuppressive drugs.

As a method for preventing host immune cell infiltration into transplanted islets, microencapsulation of islets has been proposed. However, the relatively large size of microcapsules influenced the

* Corresponding author. Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, South Korea. Tel.: +82 2 880 7866; fax: +82 2 872 7864.
** Corresponding author. Tel.: +82 2 2220 2348; fax: +82 2 2220 4741.
E-mail addresses: dongyunlee@hanyang.ac.kr (D. Y. Lee), yrbyun@snu.ac.kr (Y. Byun).
[1] These authors equally contributed as corresponding authors in this work.

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.06.068

consequential limitation of mass and oxygen transports, and the size of microencapsulated islets was not suitable for transplantation via hepatic portal vein. Also, alginate, a general material for microencapsulation of islets, was known to induce the inflammation because of its impurity [6,7]. On the other hand, the surface camouflage of islets using poly(ethylene glycol) (PEG) [8–11], heparin [12,13] and chitosan derivatives [13] have been proposed as promising methods for preventing immune responses. Cabric et al. reported that modification of islet surface using heparin protected the islet acute immune reaction by innate immune system in an intraportal islet transplantation [12,14]. Teramura et al. reported surface modified islet using poly(ethylene glycol)-phospholipid conjugates (PEG-lipid) for improvement of graft survival in an intraportal transplantation. It was reported that the survival time of PEG-lipid grafted islets was slightly longer than the control islets when the recipient was transplanted with 500 islets (PEG-lipid grafted islets; 5.0 ± 0.9 days, control islets; 1.8 ± 1.6 days, $P < 0.01$); however, the survival time of grafted islets was not sufficiently increased [15]. Therefore, polymeric therapy like a surface modification strategy did not perfectly protect the host immune reaction

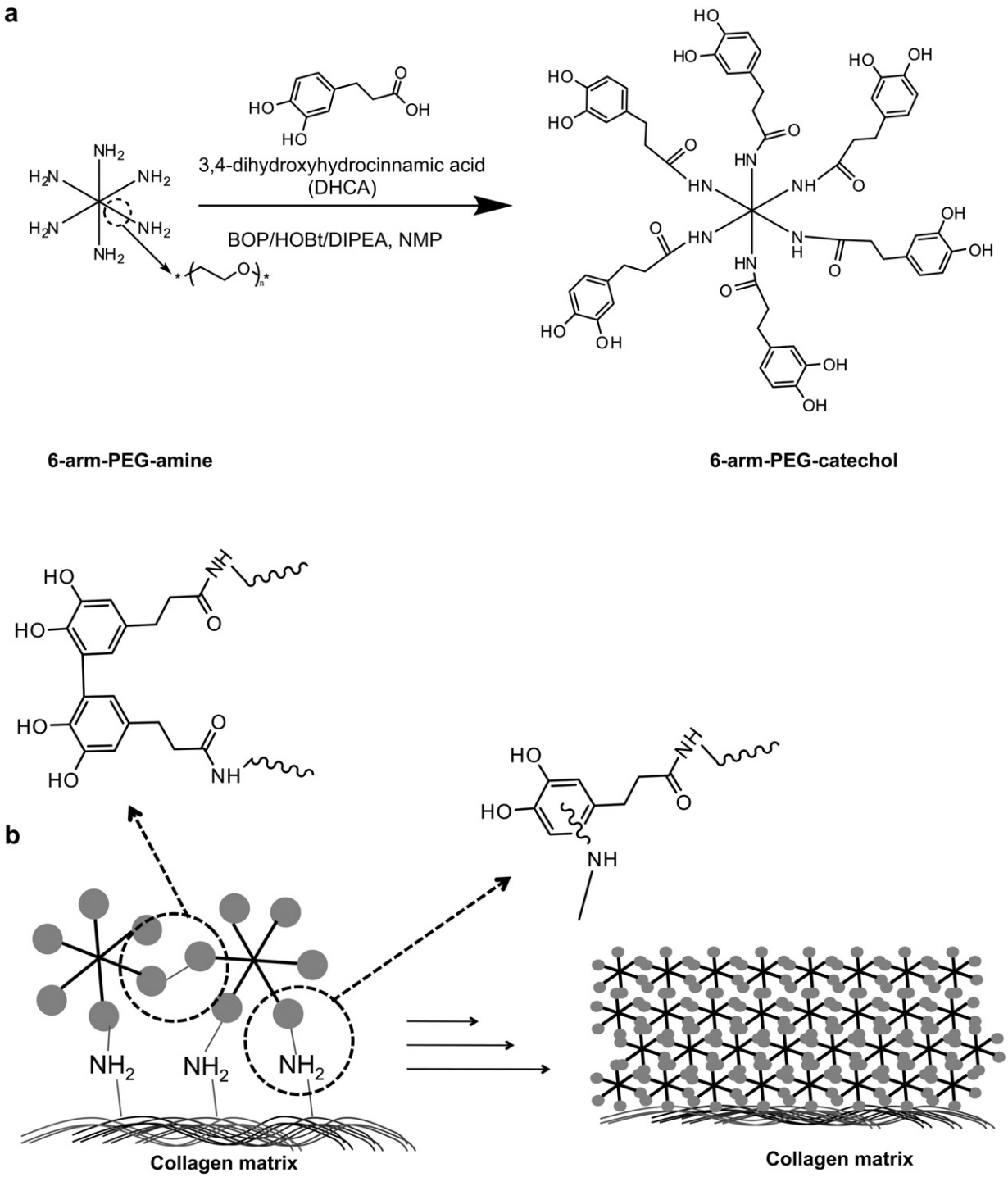

Fig. 1. (a) Synthesis of 6-arm-PEG-catechol from 6-arm-PEG-amine. (b) Schematic representation illustrating the interaction between 6-arm-PEG-catechol and collagen matrix of the islet surface.

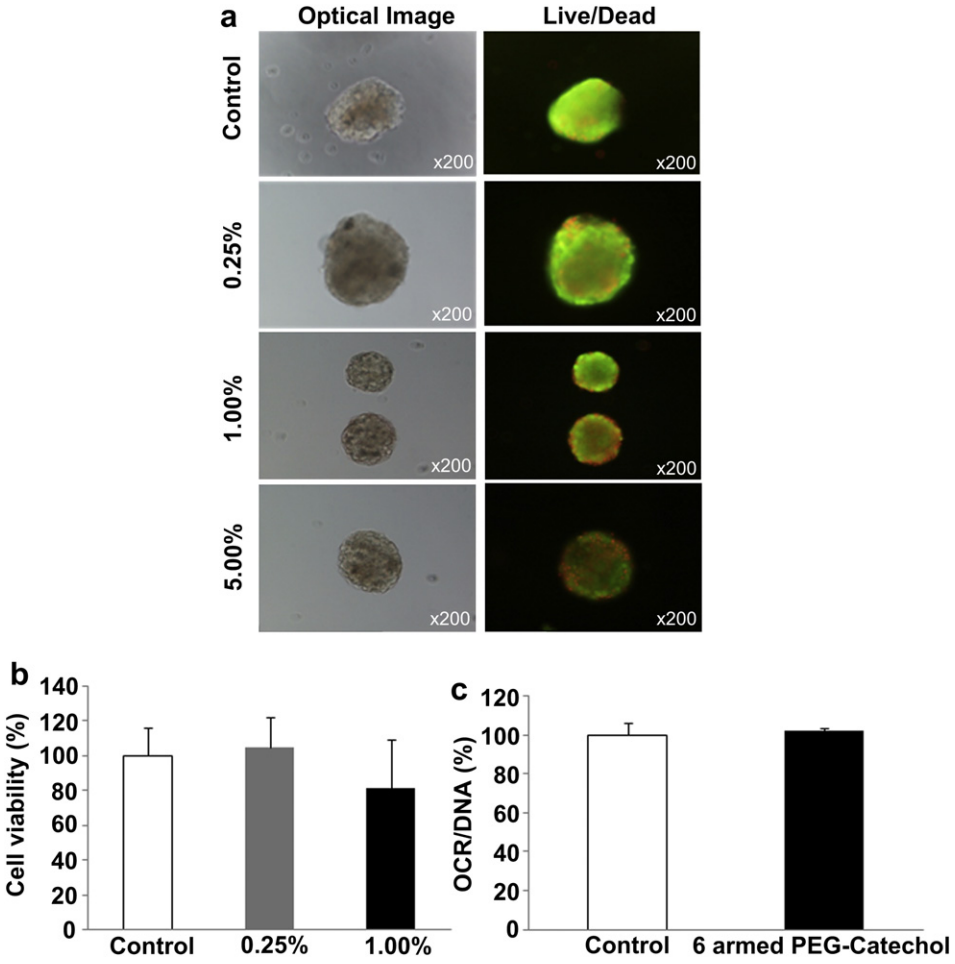

Fig. 2. (a) Live/dead cell image of unmodified (control) and 6-arm-PEG-catechol grafted islets (0.25%, 1%, and 5%) (b) The relative viability of unmodified and 6-arm-PEG-catechol grafted islets (0.25%, 1%) using CCK-8 assay ($n = 4$). (c) OCR/DNA value of unmodified and 6-arm-PEG-catechol grafted islets (0.25%) ($n = 3$).

and cytokine attack activated by immune system. We have reported on the limitation of surface camouflage of islets for protection against immune reactions and improved the survival graft time of monomethoxy-poly(ethylene glycol)-succinimidyl propionate (mPEG-SPA) grafted islets with the treatment of Cyclosporin A in allotransplantation [16].

In this study, we assumed that the catechol moiety anchored 6-arm-PEG (6-arm-PEG-catechol) could be more highly packed on the islet surface compared to the linear PEG. Hence we propose that it could effectively prevent immune cell infiltration and activation in xenotransplantation. In addition, catechol moieties, extracted from adhesive protein of mussel, were conjugated to the functional end group of 6-arm-PEG in order to effectively conjugate PEG molecules to the islet surface. Furthermore, to evaluate the ability of applying this surface camouflage system to clinical study, we assume that it would be possible to expand the synergistic effect if

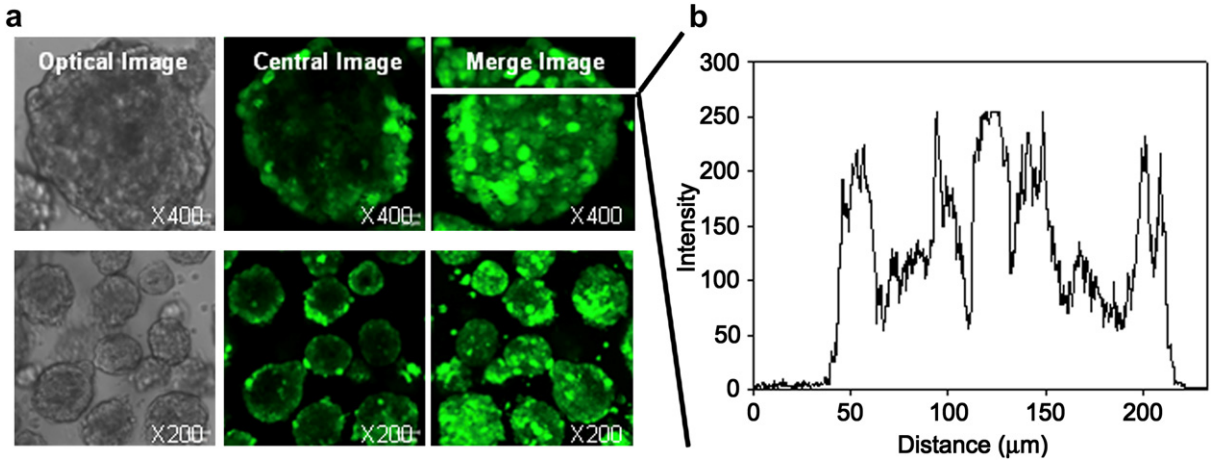

Fig. 3. (a) Confocal fluorescence images of FITC-linked 6-arm-PEG-catechol grafted islets and (b) fluorescent intensity of dark area on the merge image.

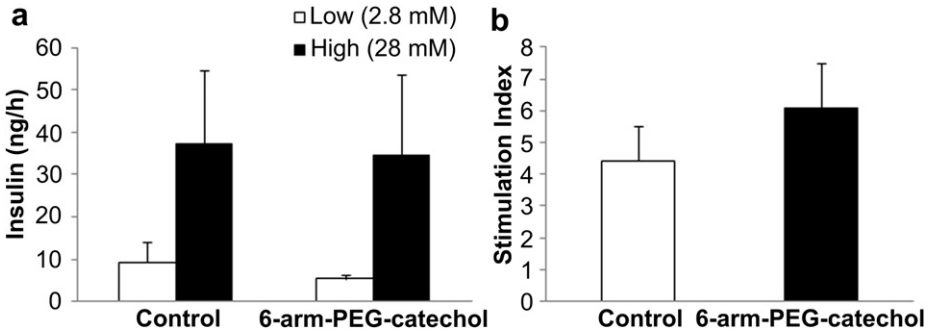

Fig. 4. (a) The glucose stimulated-insulin secretion (GSIS) of unmodified and 6-arm-PEG-catechol grafted at low (white bar, 2.8 mM) or high (Black bar, 28 mM) glucose solution for 2 h ($n = 5$) (b) Stimulation index (SI) of unmodified and 6-arm-PEG-catechol grafted islets ($n = 5$). Data were expressed as mean of ±SEM.

we use this 6-arm-PEG-catechol grafting technique in combination with low doses of the immunosuppressive drugs. In this study, a low dose of Tacrolimus (FK506), a calcineurin inhibitor, was used to prevent generation of cytokines from different immune cell activation. Anti-CD154 monoclonal antibody (MR1) was also used to inhibit the activation of $CD4^+$ T cell that is triggered by indirect immune activation pathway in xenotransplantation. Here, we suggested a convergent protocol using surface camouflage technique and low doses of immunosuppressive drugs to improve islet xenograft survival rate.

2. Research design and methods

2.1. Synthesis scheme of 6-arm-PEG-catechol

Six-arm-PEG-amine (1 g, mw 15 kDa) was dissolved in 10 ml of N-methylpyrrolidone (NMP) at 60 °C for 10 min. DHCA (3,4-dihydroxyhydrocinnamic acid, 0.8 mmol), benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP, 0.8 mmol), 1-hydroxybenzotriazole (HOBt, 0.8 mmol) and N,N-diisopropylethylamine (DIPEA, 0.8 mmol) were dissolved in 5 ml of NMP in separate vials. First, both PEG and DHCA solutions were reacted at room temperature for 6 h, followed by reacting with BOP, HOBt and DIPEA until Ninhydrin assay showed a negative result. The reacted solution was dialyzed (MWCO 8 kDa) in acidified distilled water (pH = 1–2) to prevent catechol oxidation, followed by lyophilizing.

2.2. Animals

Inbred male C57BL/6 mice (7–8 weeks of age) were used as recipients and male Sprague–Dawley (SD) rats (8 weeks of age) were used as donors. They were purchased from Orient Bio Inc. (Seongnam, South Korea) and were housed under a specific pathogen-free condition. Diabetes mellitus was chemically induced in C57BL/6 mice by a single intraperitoneal injection of 180 mg/kg of streptozocin (STZ) (Sigma, St. Louis, MO). Mice, which exhibited the glucose level over 300 mg/dl for two consecutive days, were selected as diabetic recipients for transplantation. All experimental and surgical procedures were carried out according to the guidelines of the Institute of Laboratory Animal Resources, Seoul National University (IACUC no. SNU-070822-5).

2.3. Islet isolation and 6-arm-PEG-catechol grafting

Pancreatic islets were isolated from outbred male SD rats by digestion of the pancreas using collagenase (Sigma, St. Louise, MO) and purification using discontinuous Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation. Isolated islets were then cultured for 2 days in the RPMI-1640 culture medium (Sigma) containing 10% fetal bovine serum (FBS; Sigma) at 37 °C under the humidified atmosphere containing 5% CO_2. Firstly, the isolated islets were washed twice with the Hank's balanced salt solution (HBSS; pH 8.0), and then 6-arm-PEG-catechol was grafted onto the islet surface. The 6-arm-PEG-catechol grafted islets were then suspended in 10 ml of HBSS (pH 8.0) containing 25 mg, 100 mg and 500 mg of 6-arm-PEG-catechol (0.25, 1 and 5%, w/v).

2.4. Cell viability

Viability of islets was qualitatively analyzed by Live/Dead Viability/Cytotoxicity Kit assay (Molecular Probes, Eugene, OR). Since the activity of intracellular esterase causes non-fluorescent cell-permeant calcein AM to become intensely fluorescent, the viable islets then produce an intense uniform green fluorescence. Ethidium homodimer (EthD-1) enter into the damaged islet membrane and then bind to nucleic acids, thereby producing a red fluorescence in the dead islets. Islets were cultured in the RPMI-1640 culture medium containing 10% fetal bovine serum for 3 days at 37 °C under the humidified atmosphere containing 5% CO_2. These islets were washed twice with HBSS, followed by suspending in 1 ml of HBSS containing 2 μl of 50 μM calcein AM working solution and 4 μl of the 2 mM EthD-1 for 15 min at room temperature. The islets stained with calcein AM and EthD-1 were observed under light microscope (Eclipse TE2000-S, Nikon, Japan).

The viability of islets was also quantitatively analyzed by Cell Counting Kit-8 (CCK-8) assay. The suspended islets (50 islets/well) were dispensed into a 96-well culture plate and treated with 10 μl of WST-8 [2-(2-methoxy-4-nitorphenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrozoium, monosodium salt]. The islets treated with WST-8 solution were incubated for 2.5 h and the absorbance was measured at 450 nm using a microplate reader. The viability of islets was also measured by the oxygen consumption rate (OCR) as follows: four hundreds islets were suspended in HBSS and 200 μl of suspended solution was added into a stirred titanium chamber (MicroOxygen Uptake System FO/SYSZ-P250, Plymouth meeting, Instech Laboratories, PA) at 37 °C. Oxygen partial pressure was recorded with a fluorescence based oxygen sensor (Neofox; Ocean Optics, FL) with time. The OCR data were normalized by DNA contents of each chamber.

2.5. Six-arm-PEG-catechol grafting onto the islet surface

Fluorescein isothiocyanate (FITC) linked 6-arm-PEG-catechol was immobilized to evaluate the distribution of 6-arm-PEG-catechol on the surface of islets. The molecule of FITC was reacted with 20% of amine residue in 6-arm-PEG-amine and the remaining amine residue was saturated by catechols. The immobilization of FITC-linked 6-arm-PEG-catechol was prepared by suspending freshly isolated islets in HBSS (pH 8.0) solution containing FITC-linked 6-arm-PEG-catechol

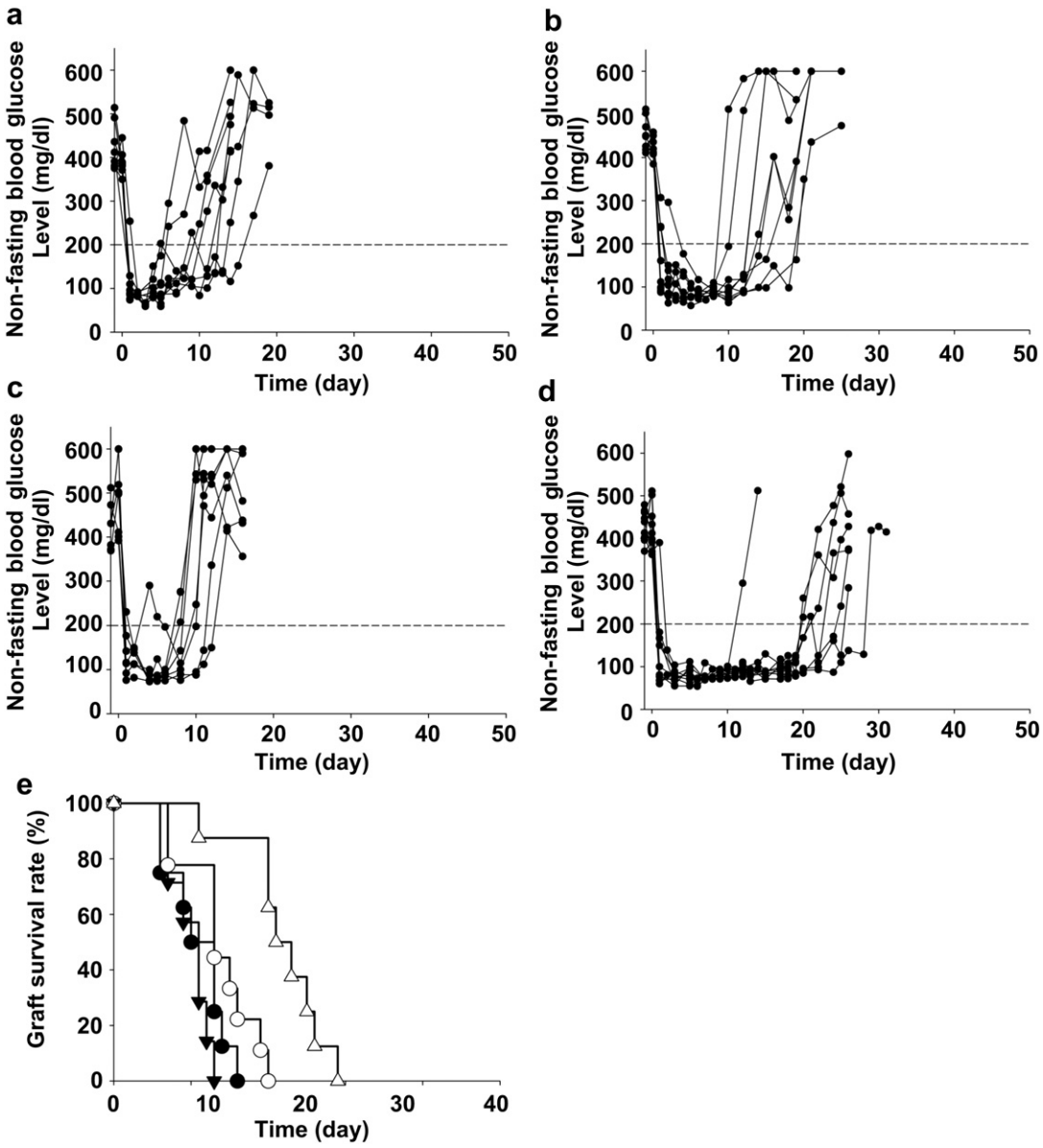

Fig. 5. Non-fasting blood glucose level after islet transplantation into diabetic mice. (a) unmodified islet recipients ($n = 8$), (b) 6-arm-PEG-catechol grafted islet recipients ($n = 9$), (c) unmodified islet recipient with FK506 treatment ($n = 7$), (d) 6-arm-PEG-catechol modified islet recipients with FK506 treatment ($n = 8$), (e) Graft survival rate of each group. (▼) unmodified islets recipients, (●) 6-arm-PEG-catechol grafted islets recipients, (○) unmodified islets recipients with FK506 treatment and (△) 6-arm-PEG-catechol grafted islets recipients with FK506 treatment.

(0.25%, w/v). FITC intensity was measured using a laser scanning confocal microscope (LSM510, Carl Zeiss, Germany).

2.6. Glucose-stimulated insulin secretion (GSIS) assay

Six-arm-PEG-catechol modified and unmodified islets were suspended in Krebs–Ringer bicarbonate buffer (KRBB; pH 7.4) containing 2.8 mM glucose, respectively. Then, 100 islets were plated into a 24-well culture plate containing a millicell culture plate insert. After pre-culture at 37 °C for 1 h under the humidified atmosphere containing 5% CO_2, the medium was exchanged with the same buffer to measure glucose stimulated insulin secretion during 2 h incubation in KRBB containing a low glucose solution (2.8 mM). Then, the medium was exchanged again with KRBB containing a high glucose solution (28 mM) for 2 h. After incubation of the islets, the secreted amount of insulin from each samples were measured using a rat/mouse insulin ELISA kit (Millipore, MA). The stimulation index (SI) value was calculated by dividing the amount of insulin secreted at a high glucose solution by that at a low glucose solution.

2.7. Xenotransplantation of rat islets into diabetic mice

Chemically induced diabetic C57BL/6 mice were anaesthetized by intraperitoneal injection with ketamin 80 mg/kg and xylazine 16 mg/kg. The left kidney of the recipient was exposed thorough the lumbar incision. Six-arm-PEG-catechol modified islets and unmodified islets (300 IEQ) were transplanted on the left kidney capsule. After islet transplantation, both body weight and non-fasting blood glucose concentrations were monitored from tail veins of mice using a portable glucometer (Super glucocard II, Arkray, Kyoto, Japan). The islet transplantation was considered a success if the blood glucose level was lower than 200 mg/dl for two consecutive days, and the transplanted islets were considered

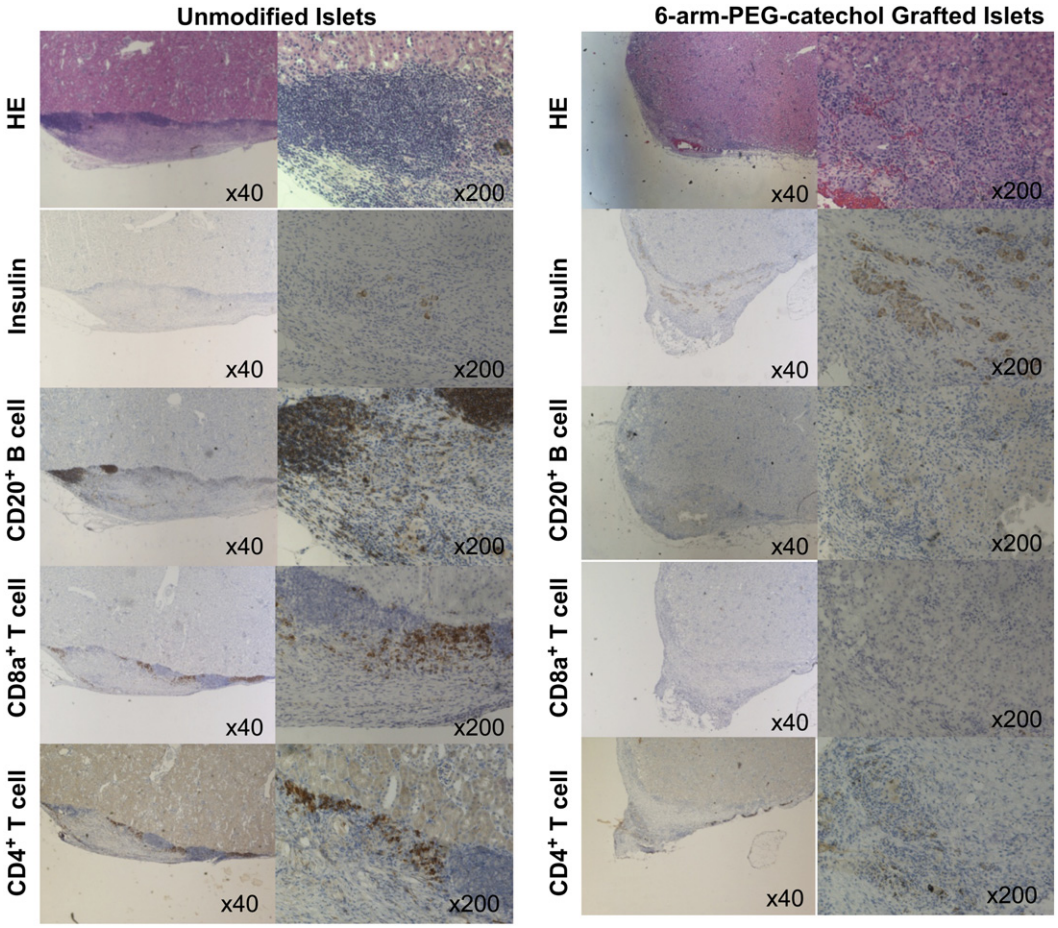

Fig. 6. Immunohistochemical analysis of transplanted islets when treated with FK506.

as rejected if the blood glucose concentration was higher than 200 mg/dl for two consecutive days.

In addition, intraperitoneal glucose tolerance test (IPGTT) using 20% glucose solution (Sigma) was performed to evaluate the glucose responsiveness of the transplanted islets at day 50 of transplantation. Examined groups were fasted at least for 6 h before the experiment with free access to water prior to the glucose tolerance test. A solution of 20% D-glucose (10 μl/g) was injected into the peritoneal cavity and the change of the blood glucose was measured from the tail vein at different time interval.

Synergistic effects of FK506 (Prograf®, Astellas Pharmaceuticals, Chicago, IL), MR1 (anti-CD154 mAb, BioXcell, West Lebanon, NH) and PEGylation on immunoprotection of transplanted islets were evaluated as follows: Recipients were daily administered intraperitoneally 0.2 mg/kg of FK506 diluted with 200 ul PBS (pH 7.4). Separately, MR1 (0.1 mg/mouse) was intraperitoneally injected into recipients on 0, 2, 4, and 6 days of post-transplantation.

2.8. Immunohistochemistry

The left kidney containing the transplanted islets was retrieved, and it was fixed in neutral 4% paraformaldehyde-phosphate-buffered saline, embedded in paraffin and sectioned at 4 μm. The tissue sections were deparaffinized in xylene and then rehydrated in serially graded alcohol. Insulin antigen retrieval consisted of heating in 10 mM citrate buffer of pH 6.0 by microwaves (5 min, 3 times, 700 W), and then cooling to room temperature for 20 min. After washing with distilled water, the slides were preincubated in 4% bovine serum albumin and dextran solution for 30 min to reduce nonspecific binding. The slides were incubated for 1 h at room temperature with mouse monoclonal anti-insulin (Abcam Inc., Cambridge, MA), anti-$CD20^+$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-$CD4^+$ (Abcam Inc.), and anti-$CD8a^+$ (BioLegend) at a 1:50, 1:40, 1:1000 and 1:100 dilution in a humidified chamber, respectively. After washing, the tissue sections were observed with a peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins in Tris–HCl buffer (Envision plus System-HRP labeled polyer; Dako, Glostrup, Denmark), which was incubated for 30 min at room temperature. Slides were washed, and the chromogen was developed for 5 min with liquid 3,30-diaminbenzidine (Dako). The slides were counterstained with Mayer hematoxylin. Negative controls were treated similarly with the exception of primary antibodies.

2.9. Statistical analysis

Survival time of transplanted islets were analyzed as median ± SEM. Cell viability and insulin secretion test analysis were expressed as mean ± S.D. Statistically analysis was carried out using the unpaired *t*-test or ANOVA one-way test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characterization of 6-arm-PEG-catechol grafted islets

Six-arm-PEG-catechol was prepared by HOBt/BOP coupling reaction between the amine groups of 6-arm-PEG-amine and the carboxylic group of 3,4-dihydroxyhydrocinnamic acid. The reaction

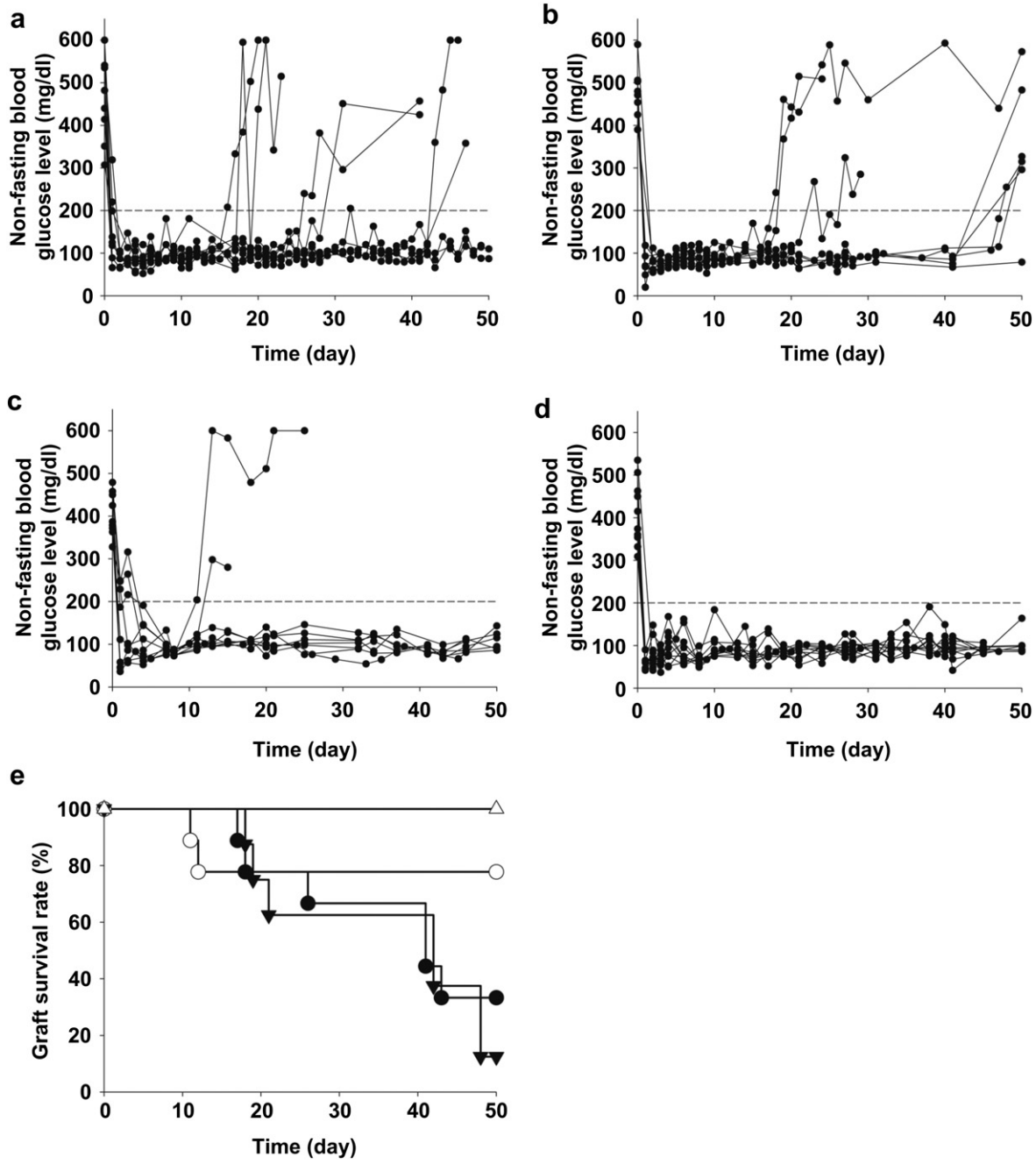

Fig. 7. Non-fasting blood glucose level after islet transplantation into diabetic mice. (a) unmodified islet recipients with MR1 treatment ($n = 9$), (b) 6-arm-PEG-catechol grafted islet recipients with MR1 treatment ($n = 8$), (c) unmodified islet recipients with FK506 treatment and MR1 ($n = 9$), (d) 6-arm-PEG-catechol modified islet recipients with FK506 and MR1 treatment ($n = 10$), (e) Graft survival rate of each group.

was monitored by Ninhydrin test, indicating that all amine groups of 6-arm-PEG molecule were reacted with 3,4-dihydroxyhydrocinnamic acid. FITC-linked 6-arm-PEG-catechol was also prepared to visualize surface modifications of islets. By Ninhydrin test and 3,4-dyhydroxyhydrocinnamic standards (A_{280}), about 20% amine groups of 6-arm-PEG-amine were coupled with FITC. The reaction between 6-arm-PEG-catechol and collagen matrix of the islet surface was illustrated in Fig. 1. The cytotoxicity of 6-arm-PEG-catechol against islets was visually evaluated using a LIVE/DEAD Viability/Cytotoxicity Kit. The endocrine cells composing the outer layer of islets were visibly dead when they were suspended in the HBSS solution containing 1 and 5% of 6-arm-PEG-catchol. Cell viability of 1% 6-arm-PEG-catechol grafted islets was 81.2 ± 21.4% when measured using CCK-8. However, islets grafted with 0.25% of FITC labeled 6-arm-PEG-catechol showed green fluorescence even at the outer layer of surface (Fig. 2A,B). Most of the single islet cells were alive after the 6-arm-PEG-catechol immobilization. The PEG incubation time was optimized as 1 h from our previous study [17]. One-hour incubation time was sufficient to cover the whole surface of islets. Therefore, for optimal PEGylation condition, the concentration of 6-arm-PEG-catechol was set at 0.25% and incubated for 1 h incubation.

The viability and functionality tests were performed using 0.25% 6-arm-PEG-catehol grafted islets. Both Cell Counting Kit-8 assay and OCR/DNA assay demonstrated that the cell viability of 6-arm-PEG-catechol grafted islets were 105.0 ± 17.2% and 101.8 ± 3.1%, compared to that of unmodified islets, respectively (Fig. 2B, C).

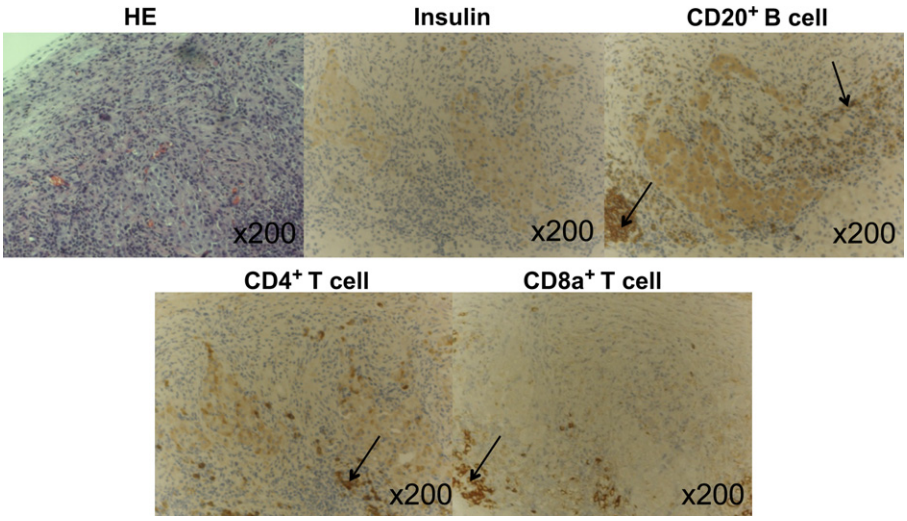

Fig. 8. Immunohistochemical analysis of transplanted 6-arm-PEG-catechol grafted islets when treated with FK506 and MR1.

There was no statistical difference in the cell viability between 6-arm-PEG-catechol grafted islets and unmodified islets. Therefore, it was confirmed that the surface camouflage using 6-arm-PEG-catechol did not affect the viability of islets.

To confirm the degree of cell coverage, the distribution profile of FITC-linked 6-arm-PEG-catechol grafted islets was observed under confocal laser scanning microscopy (CLSM). As shown in Fig. 3A, 6-arm-PEG-catechol was mostly grafted on the surface of islets. The optimized PEG concentration and incubation time prevented the diffusion of PEG molecule inside the islets. The fluorescence intensity of projection image indicated that the FITC-linked 6-arm-PEG-catechol was evenly distributed to the whole surface of islets (Fig. 3B). However, the surface of islets was not smooth and some PEG aggregates were found on the surface and this is due to the reactivity of catechol moieties unbound to the collagen matrix of islets. They reacted with adjacent catechol moieties and formed aggregates on the surface of islets.

3.2. Glucose-stimulated insulin secretion from 6-arm-PEG-catechol grafted islets

The glucose-stimulated insulin secretion was measured to evaluate the ability of 6-arm-PEG-catechol grafted islets to control the insulin release in response to glucose. There was no significant difference in an insulin release between unmodified islets and 6-arm-PEG-catechol grafted islets. At a high glucose concentration, the secretion rates of insulin from 50 unmodified islets and 50 6-arm-PEG-catechol grafted islets were 37.29 ± 17.55 ng/h and 34.50 ± 19.28 ng/h, respectively (Fig. 4A). The stimulation index (SI) values of unmodified islets and 6-arm-PEG-catechol grafted islets were 4.4 ± 1.1 and 6.1 ± 1.4, respectively (Fig. 4B). These SI values were not statistically different, and this result indicated that the grafted 6-arm-PEG-catechol did not affect the functionality of islets for releasing insulin.

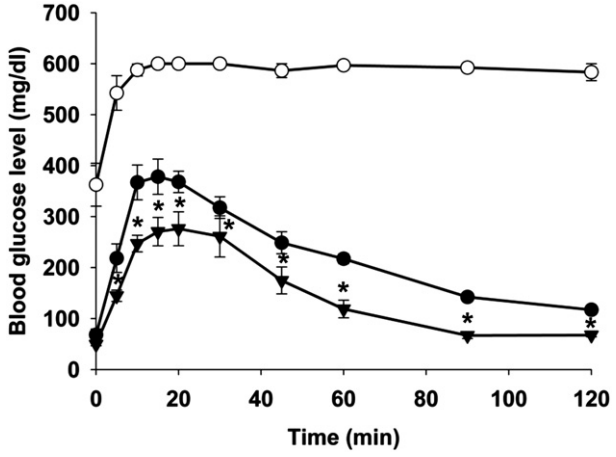

Fig. 9. The intraperitoneal glucose tolerance test (IPGTT) of normal mice (●) ($n = 6$), diabetic mice (○) ($n = 6$), 6-arm-PEG-catechol grafted islets recipients treated with FK506 and MR1 (▼) 50 days after transplantation. Data were expressed as mean ± SEM ($n = 10$), (*$P < 0.01$ vs. diabetic group, t-test).

3.3. Islet transplantation in diabetic mice

To evaluate the therapeutic potential of 6-arm-PEG-catechol grafted islets, the islets were transplanted under the left kidney capsule of chemically induced (STZ) diabetic mice, followed by measuring non-fasting blood glucose levels of recipients (Fig. 5). The median survival times (MST) of unmodified islet and 6-arm-PEG-catechol grafted islets were 10.5 ± 1.3 days and 12.0 ± 1.1 days (median ± SEM, $p < 0.05$), respectively. Although the MST of 6-arm-PEG-catechol grafted islets was slightly increased compared to that of unmodified islets, all the transplanted islets were rejected within 20 days. To verify the synergistic effect of surface camouflage using 6-arm-PEG-catechol and FK506, 0.2 mg/kg of FK506 was daily administrated after 6-arm-PEG-catechol grafted islets or unmodified islets were transplanted. FK506 did not improve the MST of unmodified islets, and the islets in all of the mice were completely rejected within 2 weeks (MST: 10.0 ± 2.9 days). However, when FK506 was daily administered after 6-arm-PEG-catechol grafted islets were transplanted, MST of the islets was increased up to 21.0 ± 1.9 days. Thus, the results of the statistical analysis ($p < 0.01$) show that the treatment of FK506 for 6-arm-PEG-catechol grafted islets could increase the survival time of islets unlike the unmodified islets in xenotransplantation.

On day 15 of transplantation, the left kidney containing transplanted islets was nephrectomized and immunostained to analyze the secretion of insulin and recruitment of immune cells (Fig. 6). In the case of unmodified islets, most of islets disappeared, and a little amount of secreted insulin was detected. In contrast, many $CD4^+$, $CD8a^+$ and $CD20^+$ positive immune cells were detected at the graft transplanted site. On the other hand, tissue containing 6-arm-PEG-catechol grafted islets had higher amounts of islet cell mass and insulin than the control. $CD8a^+$ and $CD20^+$ positive immune cells

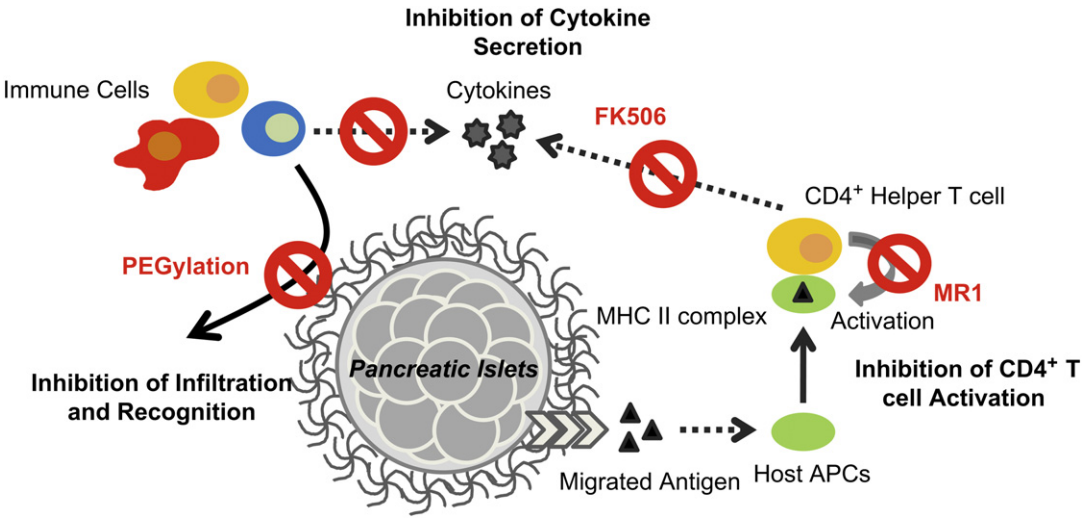

Fig. 10. Illustration of convergent protocol for islet immunoprotection.

were rarely detected around the transplanted site. However, $CD4^+$ T cells were still detected near the transplanted site, explaining that the grafted 6-arm-PEG-catechol could not attenuate the T cell activation. Therefore, $CD4^+$ T cells might be the main reason why FK506 could not significantly prolong the survival time of 6-arm-PEG-catechol grafted islets in xenotransplantation.

To prevent the $CD4^+$ T cell activation, which is triggered by migrated antigen from transplanted islets, anti-CD154 monoclonal antibody (MR1) was additionally administered (Fig. 7). MR1 could increase the survival time of transplanted islets in each case of unmodified islets and 6-arm-PEG-catechol grafted islets. Both MR1 and FK506 further increased the survival time of transplanted islets. Upon injection of MR1 and FK506, all recipients receiving 6-arm-PEG-catechol grafted islets maintained the normal glucose level until nephrectomy at day 50 of transplantation. When 6-arm-PEG-catechol grafted islets were treated with MR1 and FK506, the blood glucose level was more stably maintained in the normal range without any fluctuation although the MST of these islets was not statistically different from that of the unmodified islets.

As shown in Fig. 8, large mass of insulin positive cells was detected, and $CD4^+$ and $CD8a^+$ T cells and $CD20^+$ B cells were rarely detected around the transplant site when 6-arm-PEG-catechol grafted islets were transplanted with administering MR1 and FK506.

IPGTT confirmed glucose responsiveness of the 6-arm-PEG-catechol grafted islets treated with FK506 and MR1 at day 50 of transplantation (Fig. 9). After a high dose of glucose was administered to the normal mice, the blood glucose level was slightly increased and rapidly returned to the normal range within 2 h. On the other hand, the blood glucose level of diabetic mice remained greater than 500 mg/dl after 15 min of the glucose injection. In the case of 6-arm-PEG-catechol grafted islets, the blood glucose level profile was similar to that of normal mice, indicating that 6-arm-PEG-catechol grafted islet recipients treated with FK506 and MR1 had a normal glucose sensitivity until day 50 of transplantation.

4. Discussion

In this study, we developed a new convergent protocol for the xenotransplantation of pancreatic islets by surface camouflage using polymer and immunosuppressive drugs such as FK506 and MR1. Six-arm-PEG-catechol effectively covered the islet surfaces, and the catechol moiety was used to play a major role in conjugating 6-arm-PEG on the islet surface. The catechol moiety is a component extracted from *Mytilus edulis* foot protein 1 (Mefp-1), which is known as mussel adhesive proteins, and it works as a surface-independent anchor molecule [18,19]. Oligo or multimerized catechol molecules can bind to the versatile surfaces including hydrophobic, fluorine-containing materials and even extra hepatic areas [20–23]. Since the isolated pancreatic islets were fully covered by collagen matrix, 6-arm-PEG-catechol would be conjugated onto collagen matrix without their conjugation on the cell membrane under the optimized condition. If the conjugation time was increased, PEG molecules could diffuse into islets and reduce cell viability. In this study, 6-arm-PEG-catechol completely covered the surface of islets without causing any damage on islet cell viability and their functionality.

In xenotransplantation, when the survival time of 6-arm-PEG-catechol grafted islets and unmodified islets were compared, the survival time of islets was not improved by grafting of 6-arm-PEG-catechol. Although surface camouflage might inhibit the immune cell recognition and infiltration, it would not prevent the activation of immune cells and secretion of cytokines from activated immune cells. To overcome the limitation of surface camouflage technology of islets, we introduced a low dose of FK506 as a calcineurin inhibitor, which was used in what is known as the “Edmonton protocol” [1,24,25]. When a low dose of FK506 was continuously administered after the transplantation of 6-arm-PEG-catechol grafted islets, its mean survival time was prolonged twice since FK506 inhibited the secretion of cytokines by interfering with the production of IL-2 by T cells, thereby attenuating the recruitment of $CD20^+$ B cells and $CD8a^+$ T cells around the transplanted site [26], $CD4^+$ T cells, however, were still present around the transplanted site but without infiltrating, and this activation of $CD4^+$ T cells was the reason that the survival time of the transplanted islet was not significantly increased.

Finally, MR1 (co-stimulatory receptor binding molecule) was additionally administered with FK506 to the recipients that 6-arm-PEG-catechol grafted islets were transplanted. All recipients in this group maintained their normal blood glucose level up to day 50 of transplantation. In addition, 6-arm-PEG-catechol grafted islets were well preserved around the transplanted site at day 50 of transplantation without recruiting $CD20^+$, $CD4^+$, and $CD8a^+$ T cells around the 6-arm-PEG-catechol grafted islets.

Several clinical studies have reported that MR1 is a potent drug candidate for improving the islet graft survival rate. However, MR1 has not been approved for the current drug market because of its

potential for triggering thromboembolism [27,28]. In this study, only 20% of the established MR1 concentration (0.5 mg/mice) with a low dose of FK506 was used in combination to prevent host immune reactions. Therefore, the synergistic effect of surface camouflage using 6-arm-PEG-catechol and MR1 and FK506 could reduce adverse effects of the immunosuppressive drugs by enhancing their immunoprotective effects.

5. Conclusion

The newly developed protocol of the surface camouflage of pancreatic islets using 6-arm-PEG-catechol and co-administration of immunosuppressive drugs such as MR1 and FK506 would certainly be an effective combination therapy for the pancreatic islet xenotransplantation. The effects of convergent technology would be summarized as shown in Fig. 10: i) Surface camouflage using 6-arm-PEG-catechol prevented the infiltration of immune cells and inhibited the antigen recognition; ii) The low dose of FK506 alleviated the inhibition of cytokine secretion from host immune cells. iii) MR1 prevented T cell activation.

Acknowledgments

This study was supported by a grant from the World Class University (WCU) program (grant no. R31-2008-000-10103), the Converging Research Center Program (grant no. 2009-0081879) and Basic Science Research Program (grant no. 2010-0027955) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology, Republic of Korea.

References

[1] Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355:1318–30.
[2] Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230–8.
[3] Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. N Engl J Med 2002;346:580–90.
[4] European best practice guidelines for renal transplantation. Section IV: long-term management of the transplant recipient. Nephrol Dial Transplant 2002;17(4):1–67.
[5] Ayres RC, Dousset B, Wixon S, Buckels JA, McMaster P, Mayer AD. Peripheral neurotoxicity with tacrolimus. Lancet 1994;343:862–3.
[6] Kendall Jr WF, Darrabie MD, El-Shewy HM, Opara EC. Effect of alginate composition and purity on alginate microspheres. J Microencapsul 2004;21:821–8.
[7] Zhang WJ, Laue C, Hyder A, Schrezenmeir J. Purity of alginate affects the viability and fibrotic overgrowth of encapsulated porcine islet xenografts. Transplant Proc 2001;33:3517–9.
[8] Wilson JT, Cui W, Chaikof EL. Layer-by-layer assembly of a conformal nanothin PEG coating for intraportal islet transplantation. Nano Lett 2008;8:1940–8.
[9] Weber LM, Cheung CY, Anseth KS. Multifunctional pancreatic islet encapsulation barriers achieved via multilayer PEG hydrogels. Cell Transplant 2008;16:1049–57.
[10] Lee DY, Lee S, Nam JH, Byun Y. Minimization of immunosuppressive therapy after islet transplantation: combined action of heme oxygenase-1 and PEGylation to islet. Am J Transplant 2006;6:1820–8.
[11] Lee DY, Park SJ, Nam JH, Byun Y. A combination therapy of PEGylation and immunosuppressive agent for successful islet transplantation. J Control Release 2006;110:290–5.
[12] Cabric S, Sanchez J, Johansson U, Larsson R, Nilsson B, Korsgren O, et al. Anchoring of vascular endothelial growth factor to surface-immobilized heparin on pancreatic islets: implications for stimulating islet angiogenesis. Tissue Eng Part A 2010;16:961–70.
[13] Yang KC, Qi Z, Wu CC, Shirouza Y, Lin FH, Yanai G, et al. The cytoprotection of chitosan based hydrogels in xenogeneic islet transplantation: an in vivo study in streptozotocin-induced diabetic mouse. Biochem Biophys Res Commun 2010;393:818–23.
[14] Cabric S, Sanchez J, Lundgren T, Foss A, Felldin M, Kallen R, et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. Diabetes 2007;56:2008–15.
[15] Teramura Y, Iwata H. Surface modification of islets with PEG-lipid for improvement of graft survival in intraportal transplantation. Transplantation 2009;88:624–30.
[16] Yun Lee D, Hee Nam J, Byun Y. Functional and histological evaluation of transplanted pancreatic islets immunoprotected by PEGylation and cyclosporine for 1 year. Biomaterials 2007;28:1957–66.
[17] Lee DY, Yang K, Lee S, Chae SY, Kim KW, Lee MK, et al. Optimization of monomethoxy-polyethylene glycol grafting on the pancreatic islet capsules. J Biomed Mater Res 2002;62:372–7.
[18] Waite JH, Tanzer ML. Polyphenolic substance of *Mytilus edulis*: novel adhesive containing L-Dopa and Hydroxyproline. Science 1981;212:1038–40.
[19] Lee H, Scherer NF, Messersmith PB. Single-molecule mechanics of mussel adhesion. Proc Natl Acad Sci U S A 2006;103:12999–3003.
[20] Waite JH. The phylogeny and chemical diversity of quinone-tanned glues and varnishes. Comp Biochem Physiol B 1990;97:19–29.
[21] Lee H, Dellatore SM, Miller WM, Messersmith PB. Mussel-inspired surface chemistry for multifunctional coatings. Science 2007;318:426–30.
[22] Kang SM, Rho J, Choi IS, Messersmith PB, Lee H. Norepinephrine: material-independent, multifunctional surface modification reagent. J Am Chem Soc 2009;131:13224–5.
[23] Brubaker CE, Kissler H, Wang LJ, Kaufman DB, Messersmith PB. Biological performance of mussel-inspired adhesive in extrahepatic islet transplantation. Biomaterials 2010;31:420–7.
[24] Truong W, Lakey JR, Ryan EA, Shapiro AM. Clinical islet transplantation at the university of Alberta–The Edmonton experience. Clin Transpl; 2005:153–72.
[25] Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 2001;50:710–9.
[26] Yoshimura N, Matsui S, Hamashima T, Oka T. Effect of a new immunosuppressive agent, FK506, on human lymphocyte responses in vitro. II. Inhibition of the production of IL-2 and gamma-IFN, but not B cell-stimulating factor 2. Transplantation 1989;47:356–9.
[27] Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nat Med 2000;6:114.
[28] Robles-Carrillo L, Meyer T, Hatfield M, Desai H, Davila M, Langer F, et al. Anti-CD40L immune complexes potently activate platelets in vitro and cause thrombosis in FCGR2A transgenic mice. J Immunol 2010;185:1577–83.